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Effect of High-Rate Algal Ponds on Viability of *Cryptosporidium parvum* Oocysts

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The physicochemical conditions of high-rate algal ponds were responsible for a more than 97% reduction in the infectivity of *Cryptosporidium parvum* oocysts in neonatal mice. The use of semipermeable bags of cellulose showed that pH, ammonia, and/or light seems to be a major factor for the inactivation of oocysts in wastewater, supporting the importance of alga-based systems for safer reuse of treated wastewater.

Present conventional wastewater treatment is very expensive for application in rural areas and, moreover, does not guarantee the inactivation of *Cryptosporidium parvum* oocysts from sewage (3, 12, 16, 18). The high-rate algal pond (HRAP) is a low-cost wastewater treatment system designed to achieve two goals: secondary wastewater treatment and algal biomass production. The HRAP is a combination of intensified oxidation ponds and an algal reactor. Algae supply the oxygen demand for bacterial degradation of organic matter, and bacteria excrete mineral compounds that provide the algae with nutrition. HRAPs have proved effective in removing organic matter (13) and in reducing bacterial contamination (8) and the number of nematode eggs (1), but no data are available on their role in removing *Cryptosporidium* oocysts, a subject of special interest when dealing with rural wastewater. We will focus this study on the effect of the HRAP physicochemical conditions on the viability of *Cryptosporidium* oocysts as measured with a neonatal mouse infectivity model.

HRAP pilot plants. Two identical pilot plants fed with urban wastewater were used for this study (ponds A and B) (Fig. 1). The average physicochemical characteristics of the ponds during the study period are shown in Table 1.

***C. parvum* oocysts.** Oocysts were obtained from the feces of an experimentally infected lamb, purified according to the procedures of Arrowood and Sterling (2), and stored at 4°C in a 2.5% (wt/vol) aqueous potassium dichromate solution until use.

Treatment of oocysts in HRAP. Regenerated cellulose semipermeable bags with 14,000-Da porosity were used for the experiment. These bags allowed oocysts to be in contact with the small ions present in the water while reducing the effect of other mortality factors such as bacterial and/or fungal contamination or predation. The bags were filled with 50 ml of sterile water and 10^8 oocysts. Two bags were placed in each pond for 3 (pond A) and 10 (pond B) days. Two other bags without oocysts were also placed in the ponds to test the osmotic interchange through the semipermeable membrane, and two bags with oocysts were stored at 4°C in sterile water and used as a control. At the end of the hydraulic retention time (HRT), the oocysts were washed and centrifuged with distilled water. The sediment was resuspended and adjusted to obtain 5×10^5 oocysts/25 μl.

Bioassay for viability. Two hundred fifty-seven suckling mice from 21 individual litters (7 to 16 mice/litter) of NMRI mice were divided in four groups of 3- to 4-day-old mice, day 0 postinfection (p.i.). Mice in groups A (98 mice) and B (79 mice) were inoculated intragastrically with 5×10^5 oocysts recovered from pond A and B bags, respectively. Control mice (group C, 80 mice) also received the same infection dose but with oocysts preserved under control conditions. The other two litters (group D) (19 mice) were used as uninoculated mice to check that no accidental infection occurred in the litters during the experiment.

Half of the mice in each group were sacrificed by inhalation of ether 6 days p.i., and the other half were sacrificed 10 days p.i. to verify that oocysts were not able to recover their infectivity after longer times in the intestine.

The whole intestinal tract of each mouse was removed, placed in an individual glass tube with 1 ml of phosphate-buffered saline, homogenized, and diluted 1/5 in a solution of 0.16% green malachite and 1% sodium dodecyl sulfate in water. The number of oocysts per milliliter was counted at 400× using a Neubauer slide.

Statistics. Chi-square tests were used to analyze differences in percentages of infectivity between mouse groups, and Mann-Whitney U tests were used to compare physicochemical values and infection intensities between ponds.

The percentage of infectivity was calculated as the ratio of the number of infected mice to that of inoculated mice (see Table 2). The number of oocysts per milliliter of intestinal tract was used to evaluate the infection intensity, and the percentage of infection reduction was calculated as the difference between the infection intensity of group C and that of group A or B, divided by the infection intensity of group C.

The infectivity of the oocysts from the HRAP was much lower than that from the control (Table 2), this difference being highly significant ($P < 0.001$). Infectivity in group C (control) was 100% after 6 and 10 days p.i., whereas in group A (3 days HRT) it was 40 and 5% for 6 and 10 days p.i., respectively, and only 20.5 and 12.5% in group B, respectively

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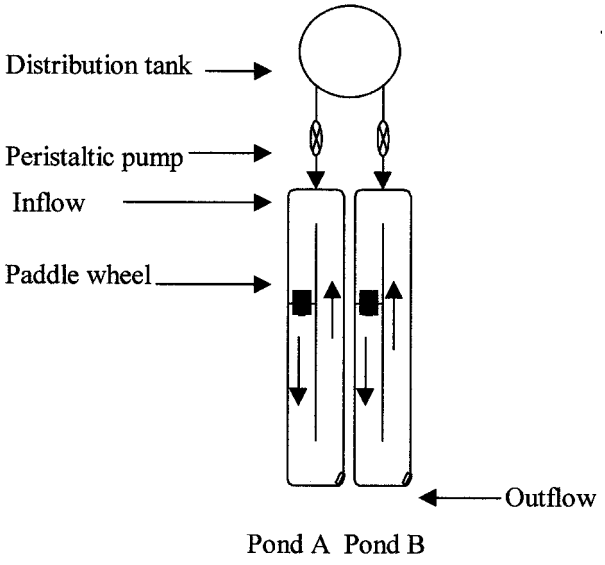

FIG. 1. Plant view of the HRAP pilot system: depth, 30 cm; length, 235 cm; area, 1.54 m^2; width, 70 cm; flow rate, 30 cm/s. HRTs were 3 days for pond A and 10 days for pond B.

(10 days HRT). The intensity of infection significantly declined ($P < 0.001$) in mice inoculated with oocysts exposed to the HRAP conditions (groups A and B).

Intestines from uninoculated mice (group D) did not contain any *C. parvum* oocysts, confirming that no accidental infections occurred during the experiment. Observed differences between pond A and pond B infectivities were not statistically significant ($P > 0.05$). This was in accordance with the lack of differences in the measured physicochemical conditions between ponds (Table 1). It means that the different HRTs assayed did not produce significant differences in the water characteristics of the ponds, and therefore, physicochemical characteristics inside the semipermeable bags were not different between pond A and pond B. Only conductivity presented significantly lower values inside the bags (Table 3).

The semipermeable bags used in the experiments excluded predation, bacterial or fungal infection, and the effect of large molecules as potential factors for oocyst inactivation. The lower conductivity values for the bags suggest that only small molecules were able to diffuse through the membrane, leaving temperature, pH, small ions (ammonia, phosphates, etc.), and light as the main factors potentially responsible for the significant reduction in oocyst infectivity.

With respect to temperature, the oocysts of *C. parvum* can retain their viability and infectivity after freezing (6) or at temperatures above 65°C (11). In our case, the water temperature was very consistent during the experiment (Table 1), suggesting that temperature was not an important factor for the inactivation of oocysts in the HRAP.

Using oocysts in semipermeable containers, Robertson et al. (15) showed that both high and low pH have a significant impact on oocyst viability. The pH in the HRAP was always higher than 8, reaching 9.5 at noon on sunny days as a consequence of the algal activity. The pH clearly influences the hydrophobic properties of the oocysts, allowing them to autoflocculate or to increase their adhesion to other particles (5).

Light also plays a significant role in the inactivation of *Cryptosporidium* oocysts, but its efficiency depends on the light type, intensity, and length of exposure. Exposure to UV light was responsible for 90 to 99% of oocyst inactivation (4, 11, 14). White light also seems to have a significant effect on *Cryptosporidium* infectivity, achieving 90% reduction in 48 h (10), and

TABLE 1. Physicochemical characteristics of HRAP during the study period

Characteristic[a]	Value ($\mu \pm$ SE) at 3 days	
	Pond A ($n = 3$)	Pond B ($n = 10$)
T^a (°C)	5.23 ± 0.35	4.78 ± 0.13
pH	8.70 ± 0.20	8.85 ± 0.04
DO (ppm)	14.5 ± 0.55	14.83 ± 0.21
Cond. (μS/cm)	323.50 ± 2.23	335.75 ± 0.55
CODd (mg of O/liter)	33.17 ± 1.61	32.87 ± 0.31
$N\text{-}NH_4$ (mg of N/liter)	3.81 ± 0.54	3.91 ± 0.08
$P\text{-}PO_4$ (mg of P/liter)	1.47 ± 0.15	1.42 ± 0.05

[a] T^a, ambient temperature; DO, dissolved oxygen; Cond., conductivity; CODd, dissolved organic matter.

TABLE 2. Infectivity in neonatal mice inoculated with 5×10^5 *C. parvum* oocysts after treatment in an HRAP at an HRT of 3 days (group A) and 10 days (group B)

Time p.i. and mouse group[a]	% Infectivity (no. of mice infected/no. of mice inoculated)	Intensity of infection (10^5 oocysts/ml, $\mu \pm$ SE)	% Reduction of infection
6 days			
A	40.0 (20/50)***[b]	0.31 ± 0.12***	97.1
B	20.5 (8/39)***	0.21 ± 0.13***	98.0
C	100.0 (40/40)	10.56 ± 1.28	0
D	0 (0/9)		
10 days			
A	5.0 (2/48)***	0.01 ± 0.01***	99.9
B	12.5 (5/40)***	0.11 ± 0.04***	99.9
C	100.0 (40/40)	15.59 ± 1.31	0
D	0 (0/10)		

[a] Group C, control group infected with oocysts that had been stored in a refrigerator at 4°C; group D, uninfected mice used to check for accidental infection during the experiment.
[b] ***, $P < 0.001$.

TABLE 3. Physicochemical characteristics of the water in the HRAP and in the cellulose semipermeable bags

Characteristic[a]	Value for water from:			
	Pond A (3 days)		Pond B (10 days)	
	HRAP	Liquid of semipermeable bag	HRAP	Liquid of semipermeable bag
pH	8.96	8.88	9.02	9.0
Temp (°C)	4.9	5.1	6.6	6.8
DO (ppm)	25.2	26	26.4	26.4
Cond. (μS/cm)	316	123[b]	361	187[b]
CODd (mg of O/liter)	31.57	31.41	30.76	30.24
$N\text{-}NH_4^+$ (mg of N/liter)	1.46	1.23	1.68	1.49
$P\text{-}PO_4^{3-}$ (mg of P/liter)	1.08	1.05	1.45	1.38

[a] DO, dissolved oxygen; Cond., conductivity; CODd, dissolved organic matter.
[b] $P < 0.05$ for HRAP value versus control liquid value, by Mann-Whitney U test.

sunlight clearly inactivates oocysts in comparison with dark conditions (9). In our experiment, the semipermeable bags were dangling in the HRAP reactor and sagging as a consequence of the water agitation. This means that bags were erratically moving from the surface to several centimeters under the surface during the study period and that therefore light could have affected oocyst viability.

Another potential factor influencing oocyst inactivation is ammonia (7). Due to the high-pH conditions of the HRAP, the ammonium-ammonia equilibrium tends toward the gas form. This dissolved ammonia could also have a significant role in inactivating oocysts.

Results show that inactivation of oocysts by HRAP is higher than that by conventional wastewater treatment systems. All oocysts present in the effluent of activated sludge plants are still active despite removal of more than 80% of the influent oocysts (18). Using semipermeable bags, Robertson et al. (17) showed that, after 1 week of exposure to different conventional treatments (activated sludge and trickling filters), none of the environments tested had a deleterious effect on oocyst viability except the anoxic sludge storage tank.

As with nematode eggs (1), this work shows that the physicochemical conditions commonly present in the HRAP seem to be responsible for the inactivation of more than 97% of the *C. parvum* oocysts. The absence of differences between the two retention times tested suggests that oocysts lost their infectivity in a very short time after contact with the water environment (probably less than 3 days). Our results strongly support other general data on the important role of alga-based systems as a very valuable treatment process in the reuse of treated wastewater.

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